Annual Vaccination against Influenza Virus Hampers Development of Virus-Specific CD8+ T Cell Immunity in Children[∇]

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Infection with seasonal influenza A viruses induces immunity to potentially pandemic influenza A viruses of other subtypes (heterosubtypic immunity). We recently demonstrated that vaccination against seasonal influenza prevented the induction of heterosubtypic immunity against influenza A/H5N1 virus induced by infection with seasonal influenza in animal models, which correlated with the absence of virus-specific CD8+ T cell responses. Annual vaccination of all healthy children against influenza has been recommended, but the impact of vaccination on the development of the virus-specific CD8+ T cell immunity in children is currently unknown. Here we compared the virus-specific CD8+ T cell immunity in children vaccinated annually with that in unvaccinated children. In the present study, we compared influenza A virus-specific cellular and humoral responses of unvaccinated healthy control children with those of children with cystic fibrosis (CF) who were vaccinated annually. Similar virus-specific CD4+ T cell and antibody responses were observed, while an age-dependent increase of the virus-specific CD8+ T cell response that was absent in vaccinated CF children was observed in unvaccinated healthy control children. Our results indicate that annual influenza vaccination is effective against seasonal influenza but hampers the development of virus-specific CD8+ T cell responses. The consequences of these findings are discussed in the light of the development of protective immunity to seasonal and future pandemic influenza viruses.

The recent pandemic caused by influenza A/H1N1 virus of swine origin and the pandemic threat caused by highly pathogenic avian influenza A/H5N1 viruses highlight the importance of these emerging viruses. However, the morbidity and mortality rates caused by pandemic influenza viruses may be reduced by the presence of immunity to these viruses induced by infection with seasonal influenza A viruses, so-called heterosubtypic immunity. Heterosubtypic immunity has mainly been demonstrated in animal models (18, 26, 28, 45), and there is also evidence for the presence of heterosubtypic immunity in humans (10, 12, 30). Influenza virus-specific CD8+ cytotoxic T lymphocytes (CTLs) are especially thought to contribute to heterosubtypic immunity since the majority of these cells recognize and lyse virus-infected cells that present conserved epitopes located in proteins like the nucleoprotein and the matrix protein (24, 27, 31, 39–40, 46). Furthermore, in humans the presence of cross-reactive CTLs inversely correlated with the extent of viral shedding in the absence of antibodies specific for the virus used for experimental infection, and in young children cellular immune responses correlated with protection against influenza (15, 32).

Seasonal influenza viruses are also an important cause of morbidity and mortality, especially in people who are at risk to develop complications after infection due to underlying disease. The World Health Organization (WHO) has recommended annual influenza vaccination of these subjects (44). In addition, it has been recommended in a number of countries that all healthy children older than 6 months of age be vaccinated against seasonal influenza (14, 41). Since universal influenza vaccines are currently unavailable, annual vaccination aims at the induction of immunity to circulating seasonal influenza viruses (A/H3N2, A/H1N1, and B viruses). Currently used inactivated influenza vaccines generally induce protective antibody responses against these viruses but inefficiently induce protective immunity to other influenza A virus subtypes (e.g., H5N1) and cross-reactive virus-specific CD8+ T cell responses (6, 11, 21).

Furthermore, it can be hypothesized that the use of these vaccines interferes with the induction of heterosubtypic immunity and virus-specific CD8+ T cell responses otherwise induced by natural infections, especially in children who are immunologically naïve to influenza viruses (7). We tested this hypothesis in mice and ferrets and confirmed that the use of inactivated A/H3N2 vaccines prevented the induction of heterosubtypic immunity to a lethal infection with influenza A/Indonesia/5/05 (H5N1) virus otherwise induced by infection with A/H3N2 influenza virus (4–6). The prevention of heterosub-

typic immunity by H3N2 vaccination correlated with reduced virus-specific CD8^{+} T cell responses.

Furthermore, epidemiological data obtained during the 2009 pandemic suggest that previous vaccination against seasonal influenza increased the risk of infection with the antigenically distinct influenza A/H1N1 pandemic virus in children and the risk of medically attended illness caused by this virus in adults (23, 25, 37). However, the reason for this in humans is unknown. Therefore, we wished to compare the frequency of influenza virus-specific CD8^{+} T cells in children who annually received influenza vaccination with the frequency in unvaccinated children.

To this end, we collected peripheral blood mononuclear cells (PBMCs) and plasma samples from cystic fibrosis (CF) patients and otherwise healthy children undergoing correctional surgery. Since CF patients are at risk for complications caused by influenza virus infections, annual influenza vaccination is recommended from the time of CF diagnosis onwards. PBMCs of the study subjects were tested for the presence of virus-specific T cells by intracellular gamma interferon (IFN-γ) staining, and plasma samples were tested for the presence of virus-specific antibodies against various influenza A virus strains and a number of control antigens used in the national immunization program. The results obtained in the present study give insight into the development of influenza virus-specific CD8^{+} T cell immunity in young children and the effect that annual vaccination with inactivated influenza A virus antigens has on the induction of this type of immunity.

MATERIALS AND METHODS

Study subjects. Children with CF who received inactivated influenza vaccine annually and unvaccinated children who visited the hospital to undergo correctional surgery were enrolled in this study. Inclusion criteria for CF children were age between 2 and 9 years with a first recorded vaccination against seasonal influenza viruses before or at 4 years of age and annual vaccination subsequently, no clinical signs of acute disease at the time of blood collection, no chronic treatment with immunosuppressive medications, and no laboratory-confirmed infection with influenza A/H1N1(2009) before or at the time of blood collection. Inclusion criteria for healthy control children were age between 2 and 9 years, no vaccination against seasonal influenza, no chronic treatment with immunosuppressive medications, and no clinical signs of disease at the time of blood collection. Blood samples were collected during autumn of 2009 and winter of 2009–2010. Written informed consent was obtained from parents or caretakers prior to enrollment. The study was approved by the institutional medical ethics committee (Medisch Ethische Toetsings Commissie Erasmus MC [METC]; protocol registration number MEC-2009-359, ABR number 29399).

Serology. Plasma samples of children were collected and stored at −20°C until use. The presence of antibodies against influenza A viruses was evaluated using the virus neutralization (VN) assay as described previously (16). Plasma samples were tested for the presence of antibodies against influenza A vaccine viruses from the influenza seasons from 2000 to 2010 and the influenza A/H1N1(2009) virus. To this end, influenza A/H3N2 viruses A/Panama/07/1999, A/Wyoming/3/2003, A/New York/55/2004, A/Hiroshima/52/2005, A/Wisconsin/67/2005, and A/Brisbane/010/2007, influenza A/H1N1 viruses A/New Caledonia/20/1999, A/Solomon Islands/3/2006, and A/Brisbane/059/2007, and the influenza A/H1N1(2009) virus A/Netherlands/602/2009 were inoculated in the allantoic cavity of 11-day-old embryonated chicken eggs. Allantoic fluid was harvested after 2 days, cleared by low-speed centrifugation, and stored at −80°C before use in the VN assay. Sera from ferrets infected with each influenza A virus were used as a positive control.

The plasma samples were also tested for the presence of IgG antibodies specific for various bacterial and viral vaccine antigens used in the national immunization program, including those for the agents of mumps, measles, and rubella, tetanus and diphtheria toxins, and a common viral pathogen (varicella-zoster virus), as described previously (42).

Collection of PBMCs and intracellular IFN-γ staining of stimulated PBMCs. Blood samples (maximum, 5 ml) were collected in EDTA tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), and subsequently, PBMCs were isolated by density gradient centrifugation using Lymphoprep solution (Axis-Shield PoC AS, Oslo, Norway) and then cryopreserved at −135°C until use. Thawed PBMCs were resuspended in RPMI 1640 medium (Cambrex, East Rutherford, NJ) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

PBMCs were seeded in 96-well U-bottom plates (8×10^5 cells/well) and infected with vaccine strain Resvir-9 (H3N2) with a multiplicity of infection of 3 or left untreated. After 16 h at 37°C, brefeldin A (2 μg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the cells. As a positive control, cells from each individual were also incubated with 1 μg/ml *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich, Zwijndrecht, The Netherlands) during incubation with brefeldin A. After 6 h, cells were washed, stained with fluorescence-labeled monoclonal antibodies (MAbs) CD4-Pacific Blue (BD, Alphen a/d Rijn, The Netherlands) and CD8-PeCy7 (eBioscience, San Diego, CA). To exclude dead cells in the analysis, cells were also stained with LIVE/DEAD Aqua fixable dead cell stain (Invitrogen, Breda, The Netherlands). Subsequently, cells were fixed with fluorescence-activated cell sorter (FACS) lysing solution (BD) and stored at −80°C until further processing. Cells were permeabilized with FACS permeabilizing solution (BD) and stained with MAbs CD3-peridinin chlorophyll protein and CD69-allophycocyanin (both from BD) and IFN-γ–fluorescein isothiocyanate (eBioscience). Data were acquired using a FACSCanto-II apparatus and analyzed with FACS Diva software (BD). For each well, the virus-specific CD8^{+} and CD4^{+} T cell response was determined by calculating the percentage of IFN-γ-positive (IFN-γ^{+}) cells of the CD69^{+} CD3^{+} CD8^{+} cell population (IFN-γ^{+} CD8^{+}) or CD69^{+} CD3^{+} CD4^{+} (IFN-γ^{+} CD4^{+}) cells. For cells incubated with medium or with Resvir-9, the assay was performed in duplicate. Subsequently, the influenza A virus-specific and SEB-specific CD8^{+} and CD4^{+} T cell response of each individual was calculated by subtracting the mean percentage of IFN-γ^{+} CD8^{+} or IFN-γ^{+} CD4^{+} cells from cells incubated with medium only from the (mean) percentage of IFN-γ^{+} CD8^{+} or IFN-γ^{+} CD4^{+} cells incubated with Resvir-9 or SEB.

Statistical analysis. Associations between the age of the children and the T cell responses of all groups were calculated using the Pearson correlation coefficient (r), and the significance was calculated using analysis of variance, which was also used to assess the difference in slope between groups. Furthermore, assuming a binominal distribution, the two-sided exact 95% confidence interval (CI) was calculated for seroprevalences of antibodies against influenza A/H3N2 and A/H1N1 viruses using Stata/SE software, version 11.0. The Mann-Whitney test was used to compare T cell responses of groups. Differences were considered significant at P values of <0.05.

RESULTS

Study population. Between 15 October 2009 and 5 February 2010, blood samples were collected from 27 unvaccinated children and 14 children with CF vaccinated against influenza annually. The mean age of unvaccinated control children was 5.9 years, and the median age of this group was 6.0 years (range, 2.0 to 8.8 years), while the mean age of the group of vaccinated children was 6.2 years (median, 6.6 years; range, 3.1 to 9.0 years).

Antibody responses to influenza viruses and other selected antigens. Plasma samples were tested for the presence of antibodies against influenza A/H3N2 and influenza A/H1N1 viruses by VN assay. In 24 out of 27 children (89%) of the unvaccinated group, antibodies were detected against at least one influenza A/H3N2 virus, and in 20 out of 27 children, antibodies were detected against one of the influenza A/H1N1 viruses, including the influenza A/H1N1(2009) virus. In two unvaccinated children, no antibodies were detected against either influenza A/H3N2 or influenza A/H1N1 virus.

In all vaccinated children, antibodies were detected against at least one influenza A/H3N2 and A/H1N1 virus. In 10 out of 14 (71%) plasma samples of these children, antibodies were detected against the influenza A/H1N1(2009) virus, against
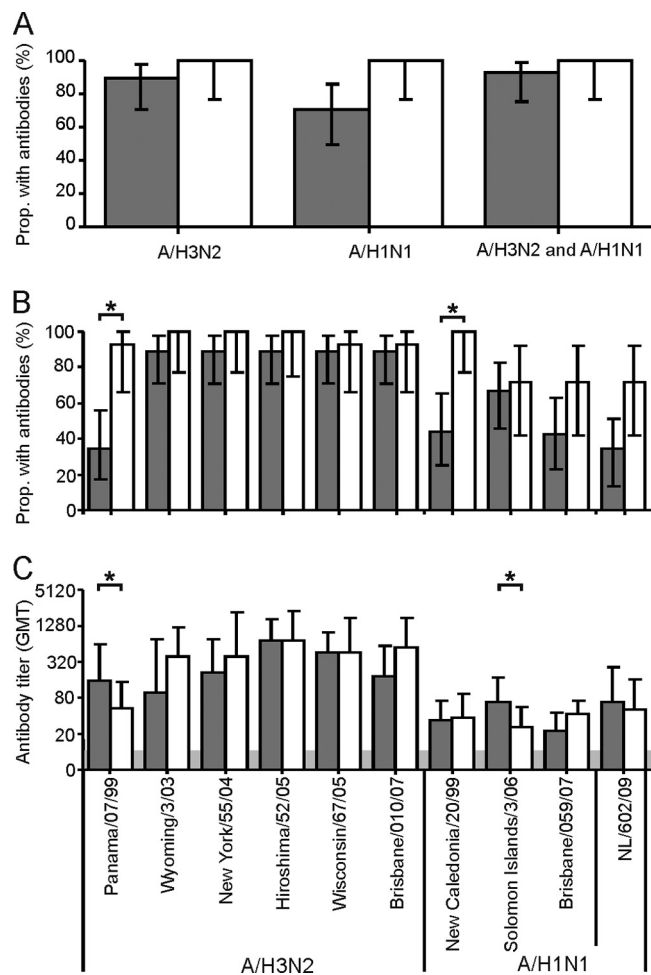
FIG. 1. Influenza A virus-specific humoral immunity. The proportion of children of the healthy unvaccinated control group (gray bars) and the vaccinated group of children with CF (white bars) with antibodies against at least one of the influenza viruses was calculated for all influenza viruses of each subtype (A) or for all indicated viruses individually (B). Bars represent percentages with 95% confidence intervals. (C) GMTs were calculated for all samples in which antibodies against the indicated viruses were detected. Bars represent GMTs with standard deviations, and the horizontal gray bar indicates the detection limit of the assay. The asterisks indicate significant differences between the two groups ($P < 0.05$).

FIG. 2. IgG antibody responses to various viral and bacterial vaccine antigens. Mean IgG antibody response ($\pm$SDs) to various viral and bacterial antigens included in the Dutch national vaccination program of the healthy unvaccinated control group (gray bars) and the vaccinated group of children with CF (white bars) for measles (A), mumps (B), rubella (C), varicella-zoster (D), diphtheria toxin (E), and tetanus toxin (F).

which they were also vaccinated (Fig. 1A). The proportion of subjects with antibodies to the relatively older strains influenza A/Panama/07/99 (H3N2) and A/New Caledonia/20/99 was significantly ($P < 0.05$) greater in the group of vaccinated children with CF than in the unvaccinated group (Fig. 1B). These differences were not observed with more recent virus strains.

Geometric mean titers (GMTs) were calculated for seropositive plasma samples to compare the magnitude of the antibody response of both groups. A significantly higher GMT was observed in children of the unvaccinated control group for both influenza A/Panama/07/99 (H3N2) and A/Solomon Islands/3/2006 (H1N1) viruses ($P = 0.04$ and $P = 0.01$, respectively). No significant differences were observed between groups for GMTs of all other viruses (Fig. 1C).

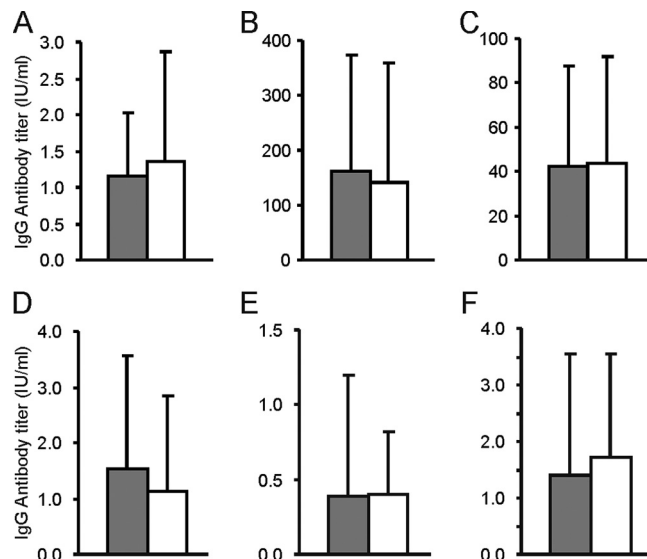The IgG antibody responses to mumps, measles, rubella, and

varicella-zoster viral antigens and the tetanus toxin and diphtheria toxin bacterial antigens were similar between the two study groups (Fig. 2A to F).

Influenza A virus-specific T cell immunity. To assess the influenza A virus-specific CD8$^+$ and CD4$^+$ T cell immunity of each study subject, PBMCs were stimulated with Resvir-9, and subsequently, intracellular IFN-γ staining was performed. The percentage of virus-specific IFN-γ^+ CD8$^+$ T cells varied from 0.00 to 2.32 for unvaccinated children, while the percentage of IFN-γ^+ CD8$^+$ T cells ranged from 0.06 to 1.56 for vaccinated children. An age-dependent increase of the virus-specific CD8$^+$ T cell response was observed in the group of unvaccinated children ($r^2 = 0.16$; $P = 0.040$; Fig. 3A), but this was not observed in the group of vaccinated children ($r^2 = 0.012$; $P = 0.714$; Fig. 3B). In addition, the age-dependent increase in virus-specific CD8$^+$ T cells in the unvaccinated group was significantly different from that in the vaccinated group ($P = 0.047$). In children older than 5 years of age, a significantly higher percentage ($P = 0.038$) of IFN-γ^+ CD8$^+$ T cells was also observed in the unvaccinated control group (mean, 0.86; standard deviation [SD], 0.67) than in the vaccinated group (mean, 0.368; SD, 0.451).

No significant age-dependent increase was observed for influenza A virus-specific CD4$^+$ T cell responses in either the unvaccinated group ($r^2 = 0.027$; $P = 0.433$; Fig. 3C) or the vaccinated group ($r^2 = 0.065$; $P = 0.379$; Fig. 3D). Furthermore, influenza A virus-specific CD4$^+$ T cell responses were similar in both groups; in unvaccinated children, the mean percentage of IFN-γ^+ CD4$^+$ T cells was 0.187 (SD, 0.169), while in the vaccinated group, the mean percentage of IFN-γ^+ CD4$^+$ T cells was 0.202 (SD, 0.276) ($P = 1.00$).

SEB-specific T cell immunity. In addition to the virus-specific T cell response, the response of CD4$^+$ and CD8$^+$ T cells
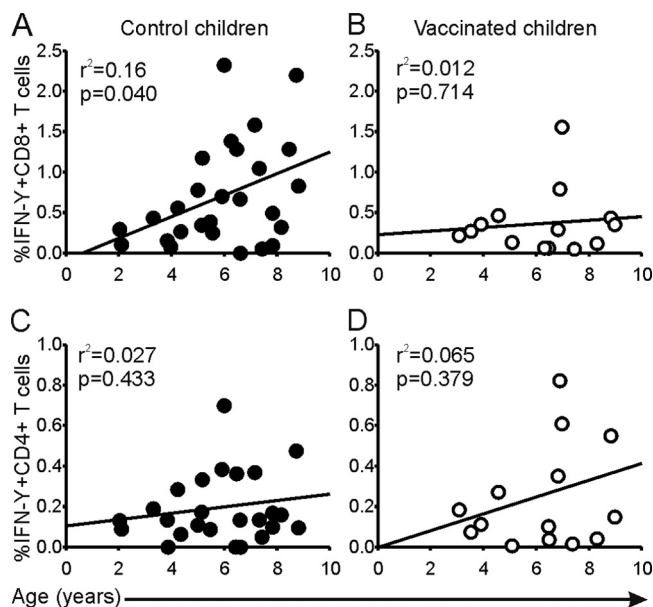
FIG. 3. Correlation between age and influenza virus-specific T cell responses. The percentage of virus-specific CD8$^+$ IFN-γ^+ T cells (A and B) and CD4$^+$ IFN-γ^+ T cells (C and D) was determined and plotted as a function of the age of the individual subjects. Each dot represents the result for an individual subject, and the correlation between all subjects of one group was calculated and is indicated by the black line. Data for both unvaccinated control children (A and C) and vaccinated children with CF (B and D) are shown. The correlation between age and the percentage of CD8$^+$ IFN-γ^+ T cells was significantly different ($P < 0.05$) between the two study groups.

FIG. 4. T cell responses to stimulation with SEB. The percentage of CD4$^+$ IFN-γ^+ T cells (A and C) and CD8$^+$ IFN-γ^+ T cells (B and D) responding to SEB was determined for each subject of the group of unvaccinated control children (A and C) and the vaccinated children with CF (B and D). The percentage of SEB-specific CD8$^+$ IFN-γ^+ T cells was also plotted against the percentage of influenza virus-specific CD8$^+$ IFN-γ^+ T cells for both the control children (E) and the vaccinated children with CF (F).

to the superantigen SEB was assessed for each subject. No age-related increase for either the SEB-specific CD4$^+$ ($r^2 = 0.039$; $P = 0.347$; Fig. 4A) or the CD8$^+$ ($r^2 = 0.024$; $P = 0.463$; Fig. 4C) T cell response was observed in unvaccinated children. Further, no significant differences were observed between the vaccinated children (mean, 1.72; SD, 1.47) and the unvaccinated children (mean, 1.37; SD, 1.01) regarding the SEB-specific CD4$^+$ T cell responses ($P = 0.558$).

In addition, no age-dependent increase of the SEB-specific CD4$^+$ T cell response was observed in vaccinated children ($r^2 = 0.148$, $P = 0.147$; Fig. 4B), while the correlation between age and the SEB-specific CD8$^+$ T cell response approached statistical significance ($r^2 = 0.229$, $P = 0.083$; Fig. 4D).

These results indicate that at a young age the percentage of SEB-specific CD8$^+$ IFN-γ+ T cells was lower in vaccinated children than unvaccinated children, while in children older than 5 years of age, no significant differences were present between groups ($P = 0.134$). Furthermore, no correlation was observed between the SEB-specific CD8$^+$ T cell response and the influenza A virus-specific CD8$^+$ T cell response in the group of unvaccinated control children ($r^2 = 0.021$, $P = 0.492$; Fig. 4E) and vaccinated children ($r^2 = 0.18$, $P = 0.12$; Fig. 4F).

DISCUSSION

In the present study, we assessed influenza A virus-specific cellular and humoral immune responses in children with CF who had been vaccinated against seasonal influenza annually and in unvaccinated cont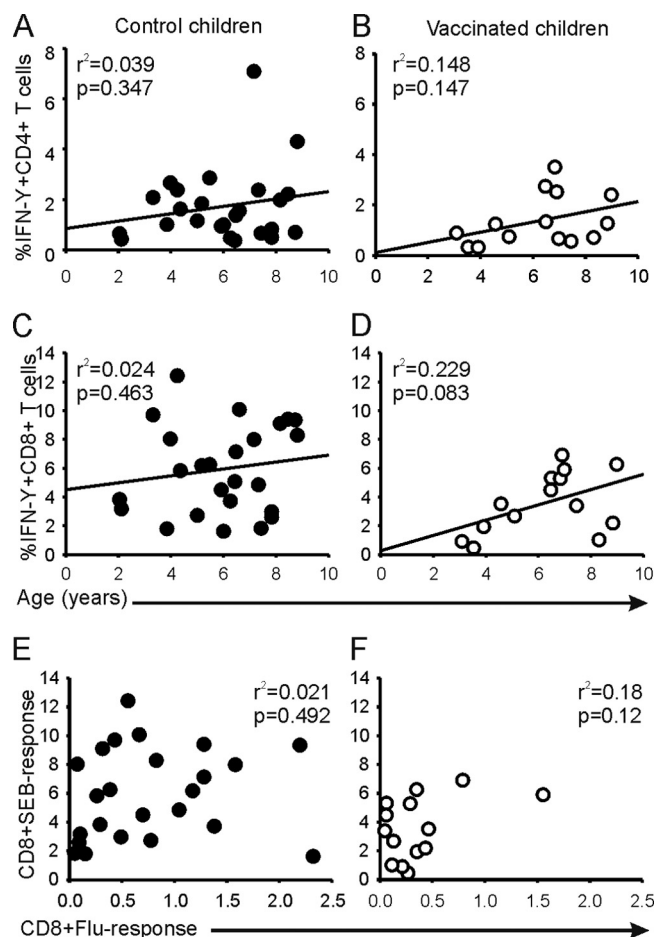rol children. The antibody recognition profile was broader in vaccinated children with CF than unvaccinated control children. No differences were observed in the development of virus-specific CD4$^+$ T cell responses. However, in unvaccinated children, an age-dependent increase in the frequency of virus-specific CD8$^+$ T cells which was not observed in vaccinated children with CF was detected. These findings are in concordance with our results in the mouse model, in which we demonstrated that vaccination against seasonal influenza A virus prevented the development of influenza A virus-specific CD8$^+$ T cell immunity otherwise induced by infection (4, 6).

It has been demonstrated previously that the majority of influenza A virus-specific CD8$^+$ T cells is directed to conserved viral proteins (29, 33). This indicates that memory CD8$^+$ T cells provoked against seasonal influenza A viruses will cross-react with other influenza A viruses, even with those of other subtypes (24, 27). Thus, vaccinated children with CF will develop lower cross-reactive virus-specific CD8$^+$ T cell responses than unvaccinated children.

The age-dependent increase in the frequency of virus-specific CD8+ T cells in the unvaccinated children most likely reflects the increase in the number of subjects who experienced an infection with an influenza virus early in life. Of interest, a similar pattern for the development of antibodies to influenza viruses was recently observed in a large seroepidemiological study performed in children ages 0 to 7 years (3). Indeed, two unvaccinated subjects without detectable antibodies to any of the influenza A viruses also had very low frequencies of virus-specific CD8+ T cells, which thus reflects a lack of exposure to influenza A virus. Maturation of the immune system may have contributed to the increased responsiveness observed in older children (8, 9, 20). However, using SEB, we were unable to demonstrate an age-dependent increase in CD4 and CD8 T cell responses to this superantigen.

In the group of CF patients vaccinated annually, the age-dependent increase in virus-specific CD8+ T cell responses was absent. Our interpretation of these findings is that vaccination efficiently induced virus-specific antibodies which protected against infection with seasonal influenza viruses to a great extent and thereby prevented the induction of virus-specific CD8+ T cell responses.

Although it would have been more ideal to compare the immune responses of unvaccinated healthy children with those of vaccinated healthy children, it is unlikely that patients with CF responded poorly because of intrinsic immunologic defects for various reasons. First, the virus-specific CD4+ T cell response of this group was comparable to that of the unvaccinated group. This confirms that the use of inactivated vaccines induced CD4+ T cell responses but not virus-specific CD8+ T cell responses, which has been demonstrated previously (21). Second, the antibody titers in the seropositive subjects were comparable between the two groups. The proportion of subjects with antibodies to older strains was higher in the group of vaccinated children. This confirms that patients with CF can be vaccinated effectively against seasonal influenza and the complications that these infections may cause in this vulnerable group of high-risk patients. Third, the CD8+ T cell response to SEB was not affected in the group of CF patients and was comparable to that in unvaccinated subjects. Finally, the antibody responses to various viral and bacterial vaccine antigens used in the Dutch national immunization program were similar for the two groups, indicating that there were no differences in the functionality of T and B cells between the groups. It is unlikely that subjects of the two groups have been exposed to viruses containing different B or T cell epitopes since the viruses causing influenza epidemics are highly homogeneous, especially in a small geographical region like The Netherlands.

Universal vaccination of healthy children is not practiced in The Netherlands, and therefore, this study group was not available. In addition, since universal vaccination of children 6 to 59 months of age has been recommended and practiced in some countries only since 2007, the long-term effects of vaccination of healthy children cannot be examined at present. Therefore, results from the present study warrant follow-up studies with larger cohorts of vaccinated and unvaccinated children in the future, especially since epidemiological data suggest that previous vaccination against seasonal influenza increased the risk of infection with pandemic influenza A/H1N1 virus in 2009 (10). Such studies would also exclude potential confounding explanations for the differences observed.

Thus, annual vaccination against influenza is effective but may have potential drawbacks that have previously been underappreciated and that are also a matter of debate (7, 22, 37). By no means do we suggest halting annual vaccination of children, especially those at high risk for complications, such as CF patients. A number of studies have demonstrated that annual vaccination reduces the morbidity and mortality caused by seasonal influenza in children and is (cost-)effective (23, 34–36). However, long-term annual vaccination using inactivated vaccines may hamper the induction of cross-reactive CD8+ T cell responses by natural infections and thus may affect the induction of heterosubtypic immunity. This may render young children who have not previously been infected with an influenza virus more susceptible to infection with a pandemic influenza virus of a novel subtype. Therefore, we argue for the development and use of vaccines that could induce broadly protective immune responses in children. For example, it has been demonstrated that live attenuated influenza vaccines induce virus-specific CD8+ T cell responses (21, 23a). In addition, it has been demonstrated that live attenuated influenza vaccines are also effective against drift variants in children (1, 2, 19). The development of broadly protective vaccines has been on the research agenda for some time, and progress has been made (13, 17, 38, 43). Young children, whether they are at high risk for influenza-associated complications or not, may especially benefit the most from these vaccines.

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